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Short communication

Detection of *Mattesia oryzaephili* (Neogregarinorida: Lipotrophidae) in grain beetle laboratory colonies with an enzyme-linked immunosorbent assay ☆

Jeffrey C. Lord *

Grain Marketing and Production Research Center, USDA, ARS, 1515 College Avenue, Manhattan, KS 66502, USA

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Abstract

An indirect sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of the neogregarine *Mattesia oryzaephili* was developed with monoclonal antibodies. It was used to screen laboratory colonies of *Oryzaephilus surinamensis*, *Cryptolestes ferrugineus*, *C. pusillus*, and *C. turcicus* from the United States, Canada, and Australia. All of the colonies except *C. turcicus* had larvae that tested positive with the percent of positives ranging from 0.2 to 83.9, but only colonies that tested positive had reported population declines. This assay will make possible epizootiological studies to assess the impact of *M. oryzaephili* on pest populations.
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Keywords: Mattesia oryzaephili; Oryzaephilus surinamensis; Cryptolestes ferrugineus; ELISA; Monoclonal antibody

1. Introduction

Mattesia oryzaephili is a neogregarine pathogen of the rusty grain beetle *Cryptolestes ferrugineus*, that was first described from the sawtoothed grain beetle *Oryzaephilus surinamensis* (Ormières et al., 1971) and is infective for several insect pests of stored products (Lord, 2003, 2006). It may be present at low prevalence rates and undetected in laboratory colonies when the practices that are used in insect culture minimize transmission and sudden outbreaks with population crashes. As with other protozoan pathogens, its impact on natural populations has not been assessed.

The epizootiology of chronic insect disease is among the most poorly understood aspects of insect pathology primarily as a consequence of the difficulty of conducting studies. Much of this difficulty can be alleviated by better tools to reduce the labor requirement and improve detection of incipient infections. Neogregarines have been linked by correlative data to reduced fitness of insect hosts and thereby implicated as population factors (Altizer and Oberhauser, 1999; Münster-Swendsen, 1991), but population data to support that implication is lacking. Epizootiological studies require a specific and sensitive means of pathogen detection that maximizes the number of hosts that can be processed. Serological methods fit this description (Fuxa, 1987). Here, I describe an enzyme-linked immunosorbent assay (ELISA) for use in epizootiological studies and give the results of tests of laboratory cultures of susceptible stored-grain beetles.

2. Materials and methods

Mattesia oryzaephili oocysts from a locally obtained culture (Lord, 2003) were produced in fourth instar *O. surinamensis* larvae and separated from macerated host tissue by centrifugation on OptiPrep (Sigma, St. Louis, MO) gradients. The oocysts were disrupted with a Mini-Beadbeater (Biospec Products, Bartlesville, OK).

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* Fax: +1 785 537 5584.
E-mail address: lord@usgmprc.ksu.edu

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Hybridomas were produced by QED Biosciences (San Diego, CA). Five spleen-donor BALB/c mice were given an initial injection of 50 μg of *M. oryzaephili* protein and five booster injections after an initial rest period of 8 weeks. Spleen cells from the animals with the highest anti-*Mattesia* titers were fused with two different myeloma cell lines. Antibody-positive parental hybridomas and subclones were evaluated by ELISA.

The specificities of anti-sera were tested by ELISA with four selected gregarine species in addition to *M. oryzaephili*. These were *Mattesia dispora*, a parasite of *Ephestia kuehniella* and *Plodia interpunctella*; *Farinocystis tribolii*, a neogregarine parasite of *Tribolium castaneum*; *Hirmocystis laemophloei* and *Gregarina cuneata*, eugregarine symbionts of *C. ferrugineus* and *Tenebrio molitor*, respectively. Five clones that reacted only with *M. oryzaephili* were selected for antibody production and were grown in Quantum Yield Basal Medium (BD Biosciences, San Jose, CA) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA) at 37 °C, 5% CO_2, and 90% relative humidity. At 3 day intervals, the cells were expanded at a 1–10 dilution and held for 2 weeks of antibody production. The supernatant was harvested by centrifugation at 1000*g* for 20 min at 4 °C and filtered through a 0.2 μm filter to remove any remaining cellular debris. The immunoglobulins were partially purified by stirring on ice with 50% ammonium sulfate for 2 h. The precipitate was collected by centrifugation 1000*g* for 30 min at 4 °C, resuspended in an equal volume of phosphate-buffered saline (PBS), and dialyzed overnight at 4 °C against 100× volume of PBS. The mouse IgG type 1 was further purified using a protein L affinity column (Pierce, Rockford, IL) according to the Manufacturer's protocol. The fractions were combined and dialyzed overnight as above. The mouse IgG concentration was estimated by the absorbance at 280 nm, aliquoted, and frozen until needed.

For indirect sandwich ELISAs, Nunc MaxiSorp 96-well plates (Nalge Nunc, Naperville, IL) were coated overnight at 4 °C with 50 μl of 1.2 μg/ml of purified polyclonal capture antibody for *Mattesia* spp. The plates were washed four times with PBS with 0.5% Tween 20 (PBST) and blocked with 1% glycine in PBST for 1 h at 37 °C. After individual homogenization of insects in 100 μl of PBST, 50 μl of each sample was added to a plate, incubated for 1 h at 37 °C, and washed. Then 50 μl of 0.75 μg/ml monoclonal antibody solution were added, and the plates were incubated for 1 h at 37 °C. Goat anti-mouse antibody conjugated to horseradish peroxidase (Sigma) was added at a concentration of 1:1000, and the plates were incubated for 30 min at 37 °C. After washing, 50 μl tetramethyl benzidine (Sigma) were added, and the plates were incubated for 20–30 min at room temperature for color development. The reaction was stopped with 1 N HCl and read at 450 nm on a BioTek EL340 microplate reader (BioTek Instruments, Winooski, VT).

All of the tested colonies were of local origin, and all have been in culture for more than 10 years except for the Montana colony, which was cultured less than 3 years ago with wild beetles added a year later. All of the Kansas colonies are maintained in one building. Two of the Kansas *C. ferrugineus* colonies have a common origin. The Montana and one of the Kansas *C. ferrugineus* colonies and the *C. turcucus* colony reported problems with population declines. Test beetles from outside of Kansas were shipped in 70% ethanol.

Ability to detect early infections was assessed by daily assay of larvae that had been exposed continuously to either 10 or 10^5 oocysts/g of diet.

3. Results and discussion

ELISAs are valuable tools for detection of the malaria parasite in mosquitoes (Wirtz et al., 1987), but we can find no previous reports of their use to detect insect pathogenic Protozoa, per se, exclusive of Microspora, which are now widely considered to be fungal. For Gregarinidea, one reason for this may be the commonality of their serological markers. Our polyclonal antibody produced against *M. oryzaephili* and many of the monoclonal antibodies cross reacted with neogregarines and eugregarines and were of no value in detecting *M. oryzaephili* in *C. ferrugineus* or other beetles that harbor eugregarines gut symbionts. This indicates that Eugregarinorida and Neogregarinorida have common dominant epitopes and confirms their systematic proximity.

Several monoclonal antibodies that were obtained reacted with *M. oryzaephili* and *M. dispora* but not the other neogregarine that was tested, *F. tribolii*. *M. dispora* is morphologically very similar to *M. oryzaephili* and has an overlapping physiological host range (Lord, 2003). *Mattesia trogodermae* is the only other member of the genus that has been found in North America. It appears to be of narrow host range, failing to infect some congeners of its type host (Schwalbe et al., 1974). Accordingly, false positives are not likely with our ELISA when testing North American insects.

The monoclonal antibody that I selected binds a protein that is weakly expressed in *M. oryzaephili* sporozoites and merozoites and strongly expressed at oocyst formation, as determined by immunofluorescence microscopy. Accordingly, the assay's ability to detect infections improves at an accelerating rate as the disease progresses. *M. oryzaephili* infection in *C. ferrugineus* larvae was detected as early as 4 days from the start of exposure to either 10 or 10^5 oocysts/g of diet. While the detected infection rate through 10 days did not exceed 30% with the low dose, it reached 100% at 6 days with the high dose. There was a positive response for one individual 2 days after exposure, possibly due to oocysts in the gut rather than actual infection.

The detection threshold was approximately 100 cells/ml of test macerate when the minimum sample-to-positive (S/P) ratio [= (OD of sample − OD of negative control)/(OD of positive control − OD of negative control)] for a positive determination was set at 0.25 (Fig. 1). Based on a sample of

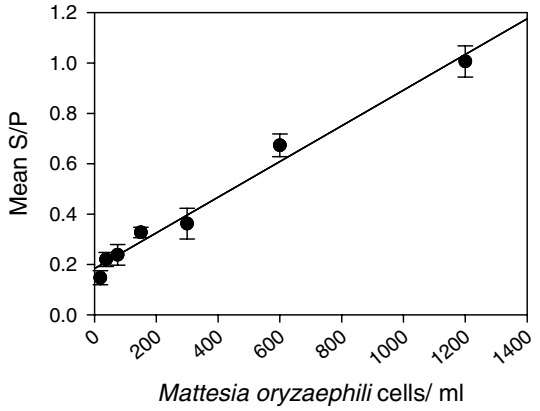

Fig. 1. The relationship between number of *Mattesia oryzaephili* oocysts and ELISA S/P ratio.

Table 1
Prevalence of *Mattesia oryzaephili* infection in *Cryptolestes* spp. laboratory colonies

Origin	*n*	Percent positive
C. ferrugineus		
US–Kansas	261	72.0
US–Kansas	261	52.5
US–Kansas	261	24.1
US–Montana	174	25.3
US–Oklahoma[a]	261	31.0
Australia	261	83.9
Canada	447	0.2
C. pusillus		
US–Kansas	261	16.5
C. turcicus		
US–Kentucky	261	0
Oryzaephilus surinamensis		
US–Kansas	261	7.6

[a] This colony was infected with a microsporidium as well as *Mattesia oryzaephili*.

245 insects, the ELISAs sensitivity was 96.7% (95% CI 93.5–99.9%), and the specificity was 92.8 (95% CI 88.3–97.3), giving a likelihood ratio of 13.4.

All seven tested *C. ferrugineus* laboratory cultures contained individuals that tested positive for *M. oryzaephili* infection (Table 1). Surprisingly, the percent of positive ranged from 0.2 to 83.9, with the higher percentages indicating colonies on the verge of collapse. The Canadian culture sample had only one positive individual, but the S/P ratio was 1.004, indicating a false positive is unlikely. Frequent transfers of eggs or adults to fresh diet can keep prevalence low and unnoticed for an extended period of time, and this could account for a prevalence of only 0.2%. The two colonies for which population declines have been noted, Montana and Kansas *C. ferrugineus*, were the only two that have been supplemented with wild insects.

We have found *M. oryzaephili* by microscopy in a small percentage of field-collected *C. ferrugineus* adults. The larvae are more susceptible than adults, and most infected larvae do not survive to complete development. Accordingly, the prevalence in larvae is likely to be much greater than that for adults. Because unexplained population crashes occur in field populations (P.W. Flinn, unpublished data), we suspect that *M. oryzaephili* may be the cause of those crashes. The ELISA will be used to track *M. oryzaephili* prevalence in field populations and to test for its presence in other stored-grain pests, such as *O. surinamensis* and *Rhyzopertha dominica*, which are susceptible to infections under laboratory conditions (Lord, 2003).

While the primary use for pathogen ELISAs is to determine field prevalence, application to cultured insects can be very useful, especially when pathogen prevalence is low in newly established colonies and contamination of rearing facilities can be avoided. The presence of undetected pathogens in laboratory insect colonies has great potential for confounding experimental work. The approach described here can be used to alert researchers of such problems as well as for assessment of field prevalence.

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